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(21) International Application Number: PCT/EP92/00716 (22) International Filing Date: 28 March 1992 (28.03.92) (30) Priority data: 9106686.0 28 March 1991 (28.03.91) GB (71) Applicant (for all designated States except US): NYCOMED AS [NO/NO]; Nycoveien 2, P.O. Box 4220, Torshov, N-0401 Oslo 4 (NO). (71)(72) Applicant and Inventor (for GB only): HOLMES, Michael, John [GB/GB]; Frank B. Dehn & Co., Imperial House, 15-19 Kingsway, London WC2B 6UZ (GB). (72) Inventors; and (75) Inventors/Applicants (for US only) : KLAVENESS, Jo [NO/NO]; Skøyen terrasse 15, N-0276 Oslo (NO). RONGVED, Pål [NO/NO]; Hovdens vei 13, N-1454 Hellvik (NO). STRANDE, Per [NO/NO]; Nordengveien 78A, N-0755 Oslo (NO).		(74) Common Representatives: HOLMES, Michael, John et al.; Frank B. Dehn & Co., Imperial House, 15-19 Kingsway, London WC2B 6UZ (GB). (81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), NO, SE (European patent), US. Published <i>With international search report.</i>
(54) Title: IMPROVEMENTS IN OR RELATING TO CONTRAST AGENTS (57) Abstract Ultrasound contrast agents comprising microbubbles of gas or a gas precursor encapsulated in a protein shell, e.g. of human serum albumin, the protein being crosslinked with biodegradable crosslinking groups, exhibit stability <i>in vivo</i> upon administration so as to permit ultrasound visualisation while allowing rapid subsequent elimination from the system.		

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"Improvements in or relating to contrast agents"

5 This invention relates to novel contrast agents, more particularly to new gas-containing or gas-generating contrast agents of use in diagnostic ultrasonic imaging.

10 It is well known that ultrasonic imaging comprises a potentially valuable diagnostic tool, for example, in studies of the vascular system, particularly in
cardiography, and of tissue microvasculature. A variety
15 of contrast agents has been proposed to enhance the acoustic images so obtained, including suspensions of solid particles, emulsified liquid droplets, gas bubbles and encapsulated gases or liquids. It is generally
20 accepted that low density contrast agents which are easily compressible are particularly efficient in terms of the acoustic backscatter they generate, and considerable interest has therefore been shown in the preparation of gas-containing and gas-generating systems.

25 Initial studies involving free gas bubbles generated in vivo by intracardiac injection of physiologically acceptable substances have demonstrated the potential efficiency of such bubbles as contrast
agents in echocardiography; such techniques are severely
30 limited in practice, however, by the short lifetime of the free bubbles. Interest has accordingly been shown in methods of stabilising gas bubbles for echocardiography and other ultrasonic studies, for example using emulsifiers, oils, thickeners or sugars.

35 WO 80/02365 discloses the use of gelatin encapsulated gas microbubbles for enhancing ultrasonic

images. Such microbubbles do not, however, exhibit adequate stability at the dimensions preferred for use in echocardiography (1-10 μm) in view of the extreme thinness of the encapsulating coating.

5

EP-A-0327490 discloses, inter alia, ultrasonic contrast agents comprising a microparticulate synthetic biodegradable polymer (e.g. a polyester of a hydroxy carbonic acid, a polyalkyl cyanoacrylate, a polyamino acid, a polyamide, a polyacrylated saccharide or a polyorthoester) containing a gas or volatile fluid (i.e. having a boiling point below 60°C) in free or bonded form. Emulsifiers may be employed as stabilisers in the preparation of such agents, but such emulsifiers do not chemically interact with the polymer.

15

US-A-4774958 discloses the use of microbubble dispersions stabilised by encapsulation in denatured protein, e.g. human serum albumin (HSA). Such systems permit the production of microbubble systems having a size of e.g. 2-5 μm but still do not permit efficient visualisation of the left heart and myocardium.

20

Other ultrasound contrast agents using proteins as encapsulating agents have been described in the literature, for example in EP 0359 246 (Molecular Biosystems), US 4,832,941 (Max-Planck Gessellschaft), US 4,844,882 (Molecular Biosystems), WO 84/02838 (Feinstein), US 4,572,203 (Feinstein), EP 0077 752 (Schering), US 4,747,610 (The Regents of the University of California), WO 80/02365 (Rasor), US 4,774,958 (Feinstein), US 4,718,433 (Feinstein), EP 0224 934 (Feinstein).

30

The only protein-based ultrasound contrast agent under commercial development consists of a suspension of gas-filled albumin, Albunex®, prepared by sonication of

35

a solution of albumin.

Albumin based ultrasound contrast agents are described in the following publications:-

- 5
- Feinstein et al. in Circulation 78S, 565 (1988),
Reisner et al. in Circulation 78S, 565 (1988), Dick et
al. in Circulation 78S, 565 (1988), Armstrong et al. in
Circulation 78S, 565 (1988), Desir et al. in Circulation
10 78S, 566 (1988), Heidenreich et al. in Circulation 78S,
566 (1988), Keller et al. in Circulation 78S, 567
(1988), Barnhart et al. in Contrast Media Research
(1989), Silverman et al. in Circulation 80S, 369 (1989),
Silverman et al. in Circulation 80S, 349 (1989), Segar
15 et al. in Clin.Res. 37, 294 (1989), Heidenreich et al.
in Circulation 80S, 370 (1989), Reiser et al. in
Circulation 80S, 370 (1989), Heidenreich et al. in
Circulation 80S, 566 (1989), Shandas et al. in
Circulation 82, 95 (1990), Geny et al. in Circulation
20 82, 95 (1990), Ten-Cate et al. in Eur Heart J. 19, 389
(1989), Feinstein et al. in Echocardiography 6, 27
(1989), Zotz et al. in Eur Heart J. 11, 261 (1990), Ten-
cate et al. in Eur Heart J. 11, 261 (1990), Barnhart et
al. in Invest Radiol 25S, 162 (1990), Keller et al. in
25 J. Am Soc Echo 2, 48 (1989), Bleeker et al. in J. Acoust
Soc Am 87, 1792 (1990), Feinstein et al. in J. Am. Coll.
Cardiol 16, 316 (1990), Kaul et al. in J. Am Coll.
Cardiol 15, 195 (1990), Bleeker et al in J. Ultrasound
Med 9, 461 (1990), Hilpert et al. in Radiology 173, 361
30 (1989), and Shapiro et al. in J. Am. Coll. 16, 1603
(1990).

However, as indicated above, ultrasound contrast
agents based on gas-filled protein microspheres are
35 unstable in vivo, and there is room for improvement of
such products. Segar et al. have, in Advances in
Echocardiography (September 21-22 - 1989), concluded

that batch, mixing pressure, mixing time and medium all affect the left atrium contrast with such protein based products.

5 Feinstein et al. have in J. Am. Coll. Cardiol 16,
316 (1990) published that irrespective of dose group, a
cavity opacification with albumin microspheres was seen
in the right ventricle in 88% of the injections and in
the left ventricle in 63% of the injections. Shandas et
10 al. have in Circulation 82, 95 (1990) raised questions
about the pressure related stability of gas filled
albumin microspheres and Shapiro et al. have recently
published in J. Am. Coll. Cardiol 16, 1603 (1990) lack
of ultrasound myocardial contrast enhancement after
15 administration of sonicated albumin.

Feinstein has in EP 0224 934 on page 4,8 and claim
9, US 4,718,433 columns 3 and 5 and US 4,774,958 columns
3 and 5 suggested chemical denaturation to stabilize
20 albumin gas bubbles:

"The microbubbles formed from 5% albumin may, in
the alternative, be stabilized to form a
commercially, clinically usable contrast agent by
treatment with various chemical agents which
25 chemically denature, or "fix", the protein, and
derivatives thereof. Chemical denaturation of the
protein (or derivatives) may be accomplished by
either binding the protein with a protein-reactive
aldehyde, such as glutaraldehyde. For the latter
30 procedure of stabilizing the invented microbubble
contrast agent, the microbubbles may be reacted
with 0.25 grams of 50% aqueous glutaraldehyde per
gram of protein at pH4.5 for 6 hours. The treated
contrast agent is then gently and extensively
35 washed to remove as much of the unreacted
glutaraldehyde as possible."

Various denaturing chemicals or crosslinking agents for proteins have been described in the literature. (See for example Methods Enzymol 172, 584 (1989) and Chemical Reagents for Protein Modification, Volume II, page 123, CRC Press Inc.)

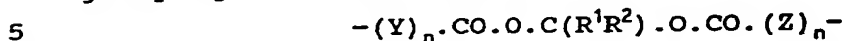
However it is important that any contrast agent should be rapidly eliminated from the subject in a short term after use, e.g. preferably having a half life of not more than 48 hours. Crosslinking by glutaraldehyde or formaldehyde may not always be effective in providing an adequate balance between stability during ultrasound visualisation and rapid elimination. The protein itself, being human serum albumin, is not rapidly degraded by vascular enzymes and reagents such as glutaraldehyde do not form readily biodegradable bonds with the protein.

The present invention is based on the concept of crosslinking the protein shells of microbubbles to introduce biodegradable linking groups, thus providing ultrasound contrast agents with adequate stability for the duration of ultrasound visualisation but sufficient biodegradability to permit rapid elimination subsequently.

According to the present invention, therefore, we provide ultrasound contrast agents comprising microbubbles of gas or a gas precursor encapsulated in a shell of protein crosslinked with biodegradable crosslinking groupings.

Biodegradable linkages which may be used include amide, imide, imine, ester, anhydride, acetal, carbamate, carbonate, carbonate ester and disulphide groups. At least one such group should preferably be present in the crosslinking grouping. In general, any

esters will be biodegradable particularly those containing the grouping -CO.O- or -O.CO.O- . One particularly useful class of biodegradable ester groupings has the structure



(where Y and Z, which may be the same or different, are -O-, -S- or -NR³-; the symbols n, which may be the same or different, are zero or 1; R¹ and R², which may be the same or different, are hydrogen atoms or carbon-
10 attached monovalent groups or together represent a carbon-attached divalent organic group; and R³ is a hydrogen atom or an organic group. Y and Z are preferably -O-. Such groups generally degrade to eliminate a compound R¹R²CO and either form carboxyl
15 groups on the residue or, in the case of carbonate esters, may eliminate carbon dioxide to form hydroxyl groups on the residue.

R¹, R² and R³ may each be a hydrocarbyl or
20 heterocyclic group, for example having 1-20 carbon atoms, e.g. an alkyl or alkenyl group (preferably having up to 10 carbon atoms), a cycloalkyl group (preferably having up to 10 carbon atoms), an aralkyl group (preferably having up to 20 carbon atoms), an acyl group
25 (preferably having up to 20 carbon atoms) or a heterocyclic group having up to 20 carbon atoms and one or more heteroatoms selected from O, S and N; such a hydrocarbyl or heterocyclic grouping may carry one or more functional groups such as halogen atoms or groups
30 of the formulae -NR⁴R⁵, -CONR⁴R⁵, -OR⁶, -SR⁶ and -COOR⁷, where R⁴ and R⁵, which may be the same or different, are hydrogen atoms, acyl groups or hydrocarbyl groups as defined for R¹ and R²; R⁶ is a hydrogen atom or an acyl group or a group as defined for R¹ or R² and R⁷ is a
35 hydrogen atom or a group as defined for R¹ or R²; where R¹ and R² represent a divalent grouping, this may for example be an alkylene or alkenylene group (preferably

having up to 10 carbon atoms) which may carry one or more functional groups as defined above. In general R^1 and R^2 are preferably hydrogen or small groups such as C_{1-4} alkyl groups.

5

The protein component can be any protein or derivative thereof including polyamino acids. Albumin, gelatin and γ -globulin are representative compounds. The protein, for instance albumin, can be obtained from biological sources, for example from human or animal blood, or produced by a lower organism using recombinant technology. A typical method for preparation of human serum albumin by fermentation is described in WO 9002808 (Delta Biotechnology Ltd.).

15

According to a further feature of the invention, we provide a process for the preparation of microbubble ultrasound contrast agents in which a gas or a gas precursor is encapsulated in a protein which is crosslinked with biodegradable crosslinking groups.

20

The crosslinking of the protein can be effected before, during or after encapsulation. It is preferred to encapsulate, e.g. by forming microbubbles, first and to effect crosslinking subsequently.

25

The crosslinking agent may be a compound of the formula (I)



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where X is a linking group containing one or more biodegradable linkages and the groups A^1 and A^2 , which may be the same or different, are functional groups reactive with proteins.

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The group X may carry further groups reactive with proteins to provide an even greater degree of crosslinking.

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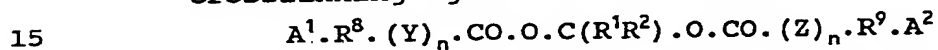
Preferably, the group X should have a chain length of not more than 30 atoms.

The group X may thus be of the form



where R^8 and R^9 , which may be the same or different, are divalent organic groups, for example alkylene or alkylidene groups having 1-12 carbon atoms, which may carry groups reactive with proteins and/or further inert groups, and the group E is an ester grouping, for example of the formula $-O.CO-$, $-O.CO.O-$ or $-(Y)_n.CO.O.C(R^1R^2).O.CO.(Z)_n-$ as defined above.

Crosslinking agents of the formula



where A^1 , A^2 , R^1 , R^2 , R^8 , R^9 , n , Y and Z have the above meanings may be prepared by reaction of an acid of the formula $A^1.R^8.(Y)_n.CO.OH$ or a form thereof in which A^1 and any other reactive groups are protected (or a functional derivative thereof) with a compound of the formula $L^1.C(R^1R^2).L^2$ where L^1 is a leaving group such as a halogen atom or mesyloxy or tosyloxy and L^2 is a group as defined for L^1 (giving a symmetrical di-ester) or a group of the formula $-O.CO.(Z)_n.R^9.A^2$ or a protected form thereof, if necessary followed by deprotection. The functional derivative of the acid may for example be a salt, e.g. the potassium salt. The reaction will normally be carried out in solution, for example in a polar solvent such as dimethylformamide. Protecting groups for A^1 and A^2 may be those conventional in the art. Preferred protecting groups for aldehydes include acetals, e.g. cyclic acetals such as dioxolan.

The compound $L^1.C(R^1R^2).O.CO.(Z)_n.R^9.A^2$, where L^1 is halogen, may be prepared from $R^1R^2.CO$ by reaction with a compound of the formula $Hal.CO.(Z)_n.R^9.A^2$ (where Hal represents a halogen atom) in the presence of a base

such as pyridine.

Apart from aldehyde groups, which are preferred, the groups A¹ and A² may be activated carboxyl groups, such as N-hydroxysuccinimidyl groups (especially water solubility-enhanced sulphonated N-hydroxysuccinimidyl derivatives), imidoesters, halo-nitroaryl groups, nitrene precursor groups such as azidophenyl, carbene precursor groups, ketone groups, isothiocyanate groups etc.

Any biocompatible gas may be employed in the contrast agents of the invention, for example air, nitrogen, oxygen, hydrogen, nitrous oxide, carbon dioxide, helium, argon, sulphur hexafluoride and low molecular weight optionally fluorinated hydrocarbons such as methane, acetylene or carbon tetrafluoride. The gas may be free within the microbubble or may be trapped or entrained within a containing substance. The term "gas" as used herein includes any substance in the gaseous form at 37°C.

Gas precursors include carbonates and bicarbonates, e.g. sodium or ammonium bicarbonate and aminomalonate esters.

For applications in echocardiography, in order to permit free passage through the pulmonary system and to achieve resonance with the preferred imaging frequency of about 0.1-15 MHz, it may be convenient to employ microbubbles having an average size of 0.1-10 µm, e.g. 1-7 µm. Substantially larger bubbles, e.g. with average sizes of up to 500 µm, may however be useful in other applications, for example gastrointestinal imaging or investigations of the uterus or Fallopian tubes.

As indicated above the microbubbles may be

stabilised by incorporation of particulate material together with the encapsulated gas. Such particles include, for example, silica and iron oxide. The preferred particle size for such stabilising particles is in the range 1 to 500 nm, depending on the size of the microbubbles. The particles should be such that they are only partially wetted by the fluid medium used to disperse the micelles, i.e. the contact angle between the material of the particles and the fluid should be about 90 degrees.

The stabilising particles may carry functional groups which will interact with the protein to form covalent or other linkages. Colloidal silica particles may have a particle size in the range 5-50 nm and may carry silanol groups on the surface which are capable of interaction with the protein by hydrogen bonding or by forming covalent bond.

The protein may stabilize the gas or gas precursor by forming a monolayer at the interface between the liquid medium and the gas or gas precursor system, or by forming vesicles consisting of one or more bilayers containing the gas or gas precursor.

The stabilisation of the system by monolayers or the formation of the vesicles may be activated, as fully described in the literature, by sonication or even shaking of the protein material mixture in the appropriate medium, or the vesicles may be formed by any conventional liposome/vesicle-forming principle.

The stabilized microbubbles may be dried or freeze-dried or the non-aqueous phase may be evaporated. The resulting dried system may be resuspended in any physiological acceptable solvent such a saline or phosphate buffer, optionally using a suspending or

emulsifying agent.

A gas entrapped system may be obtained by using a gas precursor or the gas itself may be entrapped. The gas may be entrapped into the amphiphile mixture simply by vigorously shaking the mixture in the presence of air, i.e. creating a gas-in-liquid emulsion as described in US-A-4684479. Another well established method, described i.e. in US-A-4774958 for creating a gas-containing bubble is by sonication of the mixture in the presence of air. Another well known method is passing the gas through a syringe into the mixture of the protein and the liquid. As described in US-A-3900420 the microgas-emulsion may be created by using an apparatus for introducing gas rapidly into a fast-flowing liquid. A region of low pressure is created in a liquid containing the protein material. The gas is then introduced to the region of low pressure and the gas-in-liquid system is obtained by pumping the liquid through the system.

By using the principle of electrolysis it is possible to generate the gas to be entrapped directly in a container containing the protein material. The electrolytes necessary for the electrolysis may even help to further stabilize the protein material. An aqueous solution containing electrolytes may generate hydrogen gas at the cathode and oxygen at the anode. The electrodes may be separated by a salt bridge. On adding hydrazine nitrogen gas may be generated at the anode. Using the Kolbe reaction, one may also generate CO₂ from carboxylic acids using electrolysis.

As described above, microbubbles may be obtained by forming liposomes or vesicles consisting of one or more bilayers. These vesicles may be formed at elevated pressure conditions in such a way that the gas is

entrapped in the vesicles.

In one procedure according to the invention, encapsulation is effected by agitation or sonication of the protein in an aqueous medium to yield a protein foam which is dried and thereafter suspended in a solution of the crosslinking agent in a polar organic solvent (e.g. a sulfoxide such as dimethyl sulfoxide) which is capable of wetting the protein foam.

10

The following Examples are given by way of illustration only:

Preparation 1

15 Methylene bis (α -formylacetate)

The preparation of the starting material, the dioxolan-protected aldehyde methyl α -formylacetate, is described by T. Hosokawa *et al.* J. Org. Chem. Soc. 52, (1987) 1758-1764. The protected aldehyde (6.0 g, 3.75 mmol) is treated with a mixture of 2N aqueous potassium hydroxide and tetrahydrofuran 20:80 (v/v) at reflux for 8 hours. The pH is adjusted to 8 using diluted HCl, and the mixture is evaporated to dryness. The solid is mixed with 100 ml freshly distilled and dried dimethylformamide, and after 30 minutes at 60°C the undissolved material is filtered off. Diiodomethane (150 μ l, 1.87 mmol) is added dropwise during 5 minutes to the solution at 60°C as described in WO 89/00988 page 13 (NYCOMED AS). The precipitate is removed by filtration after stirring for 4 days, and the solvent removed at reduced pressure. The dioxolan protection is removed as described by P. A. Grieco *et al.* J. Am. Chem. Soc. 99, (1977) 5773-5780 - the residue is dissolved in tetrahydrofuran (60 ml), 5% aqueous HCl (20 ml) is added and the mixture is stirred for 20 hours at ambient temperature. The reaction mixture is evaporated to dryness under reduced pressure to yield the title

compound.

Preparation 2

Methylene dimethacrylate

- 5 A solution of potassium hydroxide (1.00 M, 40.00 ml) is added to methacrylic acid (3.44 g, 40.00 mmol) at 0°C and the solution freeze dried for 16 hours. Dry dimethylformamide (230 ml) is added and the suspension heated to 60°C under a dry nitrogen atmosphere.
- 10 Diiodomethane (1.61 ml, 20.00 mmol) is added in two portions during 10 min. and the reaction mixture left for 4 days at 60°C. The solvent is removed under reduced pressure (0.05 mm Hg), before diethyl ether (140 ml), saturated aqueous sodium hydrogen carbonate (50 ml)
- 15 and water (50 ml) are added. The aqueous layer is extracted with diethyl ether (6 x 60 ml) and the combined ether extracts washed with water (4 x 50 ml), dried (MgSO₄), and evaporated to give 2.63 g (72%) of the title compound. ¹H NMR (60 MHz, CDCl₃): δ 1.97 (2 x CH₃, m), 5.63 (2 x H-C=, m), 5.88 (CH₂, s), 6.18 (2 x H-C=, m). IR (film, cm⁻¹): 2987 (w), 2962 (w), 2930 (w), 1732 (str), 1638 (w), 1454 (w), 1315 (w), 1295 (w), 1158 (w), 1100 (str), 1012 (m), 989 (m). This product may be used
- 20 in accordance with the invention, for example to
- 25 crosslink acrylamide polymers.

Preparation 3

Methylene diacrylate

- 30 A solution of potassium hydroxide (1.00 M, 40.00 ml) is added to acrylic acid (2.88 g, 40.00 mmol) at 0°C and the solution freeze dried for 16 hours. Dry dimethylformamide (200 ml) is added and the suspension heated to 60°C under a dry nitrogen atmosphere.
- 35 Diiodomethane (1.61 ml, 20.00 mmol) is added in two portions during 10 min. and the reaction mixture left for 4 days at 60°C. The solvent is removed under reduced pressure (0.05 mm Hg), before diethyl ether (140

- 14 -

ml), saturated aqueous sodium hydrogen carbonate (50 ml) and water (50 ml) are added. The aqueous layer is extracted with diethyl ether (6 x 60 ml) and the combined ether extracts washed with water (4 x 50 ml), dried (MgSO₄), and evaporated to give 1.06 g (34%) of the title compound. ¹H NMR (60 MHz, CDCl₃): δ 5.81-6.61 (2 x CH₂ = CH-, m), 5.84 (CH₂, s). This product may be used in accordance with the invention, for example to crosslink acrylic acid and methyl acrylate polymers.

10

Preparation 4

Chloromethyl (2-methacryloyloxy)ethyl carbonate

Pyridine (0.89 ml, 11.00 mmol) is added dropwise to a solution of chloromethyl chloroformate (0.89 ml, 11.00 mmol) and 2-hydroxyethyl methacrylate (1.22 ml, 10.00 mmol) in dichloromethane (12 ml) at 0°C under a dry nitrogen atmosphere. After 21 hours at 20°C the reaction mixture is washed with hydrochloric acid (1.00 M, 10 ml), saturated aqueous sodium hydrogen carbonate (10 ml) and water (10 ml). The organic phase is dried (MgSO₄) and the solvent evaporated under reduced pressure (10 mm Hg) to give 1.97g (88%) of the title compound. ¹H NMR (60 MHz, CDCl₃): δ 1.88 (CH₃, d, J=2 Hz), 4.35 (O-CH₂-CH₂-O, m), 5.47 (H-C=, m), 5.63 (CH₂-Cl, s), 6.00 (H-C=, m).

25

Preparation 5

(2-Methacryloyloxy)ethyl methacryloyloxymethyl carbonate

A solution of potassium hydroxide (1.00 M, 5.00 ml) is added to methacrylic acid (0.43 g, 5.00 mmol) at 0°C and the solution freeze dried during 16 hours. Dry dimethylformamide (50 ml) is added and to the resulting suspension is added chloromethyl (2-methacryloyloxy)ethyl carbonate (1.11 g, 5.00 mmol). 18-Crown-6 (0.066 g, 0.25 mmol) is added as a catalyst and the reaction left under a dry nitrogen atmosphere. After 24 hours at 20°C and 6 days at 4°C the solvent is removed under

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- 15 -

reduced pressure (0.05 mm Hg) and diethyl ether (30 ml) and water (20 ml) added. The aqueous layer is extracted with diethyl ether (3 x 20 ml) and the combined ether extracts washed with water (20 ml), dried (MgSO_4) and
5 evaporated to give 1.26 g (93%) of the title compound.
 ^1H NMR (60 MHz, CDCl_3): δ 1.97 (2 x CH_3 , m), 4.38 ($\text{O}-\text{CH}_2-\text{CH}_2-\text{O}$, m), 5.53 (2 x $\text{H}-\text{C}=\text{C}$, m), 5.77 (CH_2 , s), 6.07 (2 x $\text{H}-\text{C}=\text{C}$, m).

10 Preparation 6

Ethylene bis(chloromethyl carbonate)

Pyridine (0.89 ml, 11.00 mmol) is added dropwise to a solution of chloromethyl chloroformate (1.32 ml, 14.83 mmol) and ethylene glycol (0.28 ml, 5.00 mmol) in
15 dichloromethane (10 ml) at 7°C with good stirring under a dry N_2 atmosphere. After 15 min. at 7°C and 6 hours at 20°C the reaction mixture is transferred to a separating funnel with the aid of dichloromethane (10 ml). The reaction mixture is washed with hydrochloric acid (1.00
20 M, 10 ml), saturated aqueous sodium hydrogen carbonate (10 ml) and water (10 ml). The organic phase is dried (MgSO_4) and the solvent evaporated under reduced pressure to give 1.12g (90%) of the title product. ^1H NMR (300
MHz, CDCl_3): δ 4.48 (s, $\text{O}-\text{CH}_2\text{CH}_2-\text{O}$), 5.75 (s, 2 x $\text{Cl}-\text{CH}_2-\text{O}$).
25 ^{13}C NMR (75 MHz, CDCl_3): δ 65.8 ($\text{O}-\text{CH}_2\text{CH}_2-\text{O}$), 72.2 (2 x $\text{Cl}-\text{CH}_2-\text{O}$), 153.0 (2 x $\text{C}=\text{O}$).

Preparation 7

Bis(2-chloromethoxycarbonyloxyethyl)ether

30 Pyridine (0.89 ml, 11.00 mmol) is added dropwise to a solution of chloromethyl chloroformate (1.32 ml, 14.83 mmol) and diethylene glycol (0.47 ml, 5.00 mmol) in dichloromethane (10 ml) at 7°C with good stirring under a dry N_2 atmosphere. After 10 min. at 7°C and 6 hours at
35 20°C the reaction mixture is transferred to a separating funnel with the aid of dichloromethane (10 ml). The reaction mixture is washed with hydrochloric acid (1.00

- 16 -

M, 10 ml), saturated aqueous sodium hydrogen carbonate (10 ml) and water (10 ml). The organic phase is dried (MgSO_4) and the solvent evaporated under reduced pressure (10 mm Hg) to give 1.26 g (86%) title product. ^1H NMR (300 MHz, CDCl_3): δ 3.72 (m, 2 x $\text{CH}_2\text{-O}$), 4.34 (m, 2 x $\text{CH}_2\text{-O-C=O}$), 5.71 (s, 2 x $\text{Cl-CH}_2\text{-O}$). ^{13}C NMR (75 MHz, CDCl_3): δ 67.6 (2 x $\text{CH}_2\text{-O}$), 68.5 (2 x $\text{CH}_2\text{-O-C=O}$), 72.1 (2 x $\text{Cl-CH}_2\text{-O}$), 153.2 (2 x C=O).

10 Preparation 8

1-Chloroethyl 2-methacryloyloxyethyl carbonate

Pyridine (0.89 ml, 11.00 mmol) is added dropwise to a solution of 1-chloroethyl chloroformate (1.20 ml, 11.00 mmol) and 2-hydroxyethyl methacrylate (1.22 ml, 10.00 mmol) in dichloromethane (12 ml) at 3°C under a dry N_2 atmosphere. After 15 min. at 3°C and 17 hours at 20°C the reaction mixture is transferred to a separating funnel with the aid of dichloromethane (10 ml). The reaction mixture is washed with hydrochloric acid (1.00 M, 10 ml), saturated aqueous sodium hydrogen carbonate (10 ml) and water (2 x 10 ml). The organic phase is dried (MgSO_4) and the solvent evaporated under reduced pressure to give 1.76g (74%) of the title product. ^1H NMR (60 MHz, CDCl_3): δ 1.85 (3 H, d, $J=6$ Hz, $\text{CH}_3\text{-CH}$), 1.96 (3 H, d, $J=2$ Hz, $\text{CH}_3\text{-C=}$), 5.55 (1 H, m, CH=), 6.10 (1 H, m, CH=), 6.38 (1 H, k, $J=6$ Hz, CH-CH_3).

Preparation 9

Chloromethyl 4-acryloyloxybutyl carbonate

Pyridine (0.89 ml, 11.00 mmol) is added dropwise to a solution of chloromethyl chloroformate (0.98 ml, 11.00 mmol) and 4-hydroxybutyl acrylate (1.38 ml, 10.00 mmol) in dichloromethane (12 ml) at 3°C under a dry N_2 atmosphere. After 15 min. at 3°C and 17 hours at 20°C the reaction mixture is transferred to a separating funnel with the aid of dichloromethane (10 ml). The reaction mixture is washed with hydrochloric acid (1.00

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M, 10 ml), saturated aqueous sodium hydrogen carbonate (10 ml) and water (2 x 10 ml). The organic phase is dried (MgSO_4) and the solvent evaporated under reduced pressure to give 1.76g (74%) of the title product. ^1H NMR (60 MHz, CDCl_3): δ 1.82 (4 H, m, $\text{CH}_2\text{-CH}_2$), 4.27 (4 H, m, 2 x $\text{CH}_2\text{-O}$), 5.77 (2 H, s, $\text{Cl-CH}_2\text{-O}$), 5.8-6.7 (3 H, m, CH=CH_2).

Preparation 10

10 1-Chloroethyl 4-acryloyloxybutyl carbonate

Pyridine (0.89 ml, 11.00 mmol) is added dropwise to a solution of 1-chloroethyl chloroformate (1.20 ml, 11.00 mmol) and 4-hydroxybutyl acrylate (1.38 ml, 10.00 mmol) in dichloromethane (12 ml) at 3°C under a dry N_2 atmosphere. After 15 min. at 3°C and 17 hours at 20°C the reaction mixture is transferred to a separating funnel with the aid of dichloromethane (10 ml). The reaction mixture is washed with hydrochloric acid (1.00 M, 10 ml), saturated aqueous sodium hydrogen carbonate (10 ml) and water (2 x 10 ml). The organic phase is dried (MgSO_4) and the solvent evaporated under reduced pressure to give 2.26g (90%) of the title product. ^1H NMR (60 MHz, CDCl_3): δ 1.80 (4 H, m, $\text{CH}_2\text{-CH}_2$), 1.86 (3 H, d, $J=5$ Hz, CH_3), 4.24 (4 H, m, 2 x $\text{CH}_2\text{-O}$), 5.7-6.6 (4 H, m, CH=CH_2 and CH).

Preparation 11

1-Methacryloyloxyethyl 2-methacryloyloxyethyl carbonate

1-Chloroethyl 2-methacryloyloxyethyl carbonate (1.183g, 5.00 mmol) prepared as described in Preparation 8 is added to a suspension of freeze dried potassium methacrylate (0.683 g, 5.50 mmol) and 18-crown-6 (0.066 g, 0.25 mmol) in dimethylformamide (50 ml) under a dry N_2 atmosphere. After 5 days at 20°C the solvent is removed under reduced pressure and the residue dissolved by adding dichloromethane (60 ml) and water (30 ml). After separating the phases the aqueous layer is extracted

with dichloromethane (3 x 30 ml) and the combined organic phase washed with saturated aqueous sodium hydrogen carbonate (50 ml). The organic phase is dried (MgSO₄) and the solvent removed under reduced pressure to
5 give 1.10g (77%) of the title product. ¹H NMR (60 MHz, CDCl₃): δ 1.63 (3 H, d, J=5 Hz, CH₃-CH), 1.98 (6 H, s, 2 x CH₃), 4.42 (4 H, s, O-CH₂-CH₂-O), 5.62 (2 H, m, CH=), 6.15 (2 H, m, CH=), 6.84 (1 H, k, J=5 Hz, CH-CH₃).

10 Preparation 12

Acryloyloxymethyl 4-acryloyloxybutyl carbonate

Chloromethyl 4-acryloyloxybutyl carbonate (1.183g, 5.00 mmol) prepared as described in Preparation 9 is added to a suspension of freeze dried potassium acrylate
15 (0.606 g, 5.50 mmol) and 18-crown-6 (0.066 g, 0.25 mmol) in dimethylformamide (50 ml) under a dry N₂ atmosphere. After 5 days at 20°C the solvent is removed under reduced pressure and the residue dissolved by adding dichloromethane (60 ml) and water (30 ml). After
20 separating the phases the aqueous layer is extracted with dichloromethane (3 x 30 ml) and the combined organic phase washed with saturated aqueous sodium hydrogen carbonate (50 ml). The organic phase is dried (MgSO₄) and the solvent removed under reduced pressure to
25 give 1.24g (91%) of the title product. ¹H NMR (60 MHz, CDCl₃): δ 1.82 (4 H, m, CH₂-CH₂), 4.23 (4 H, m, 2 x CH₂-O), 5.88 (2 H, s, O-CH₂-O), 5.7-6.8 (6 H, 2 x CH=CH₂).

Preparation 13

30 1-Acryloyloxyethyl 4-acryloyloxybutyl carbonate

1-Chloroethyl 4-acryloyloxybutyl carbonate (1.253g, 5.00 mmol) prepared as described in Preparation 10 is added to a suspension of freeze dried potassium acrylate
(0.606 g, 5.50 mmol) and 18-crown-6 (0.066 g, 0.25 mmol)
35 in dimethylformamide (50 ml) under a dry N₂ atmosphere. After 5 days at 20°C the solvent is removed under reduced pressure and the residue dissolved by adding

dichloromethane (60 ml) and water (30 ml). After separating the phases the aqueous layer is extracted with dichloromethane (3 x 30 ml) and the combined organic phase washed with saturated aqueous sodium hydrogen carbonate (50 ml). The organic phase is dried (MgSO₄) and the solvent removed under reduced pressure to give 1.28g (89%) of the title product. ¹H NMR (60 MHz, CDCl₃): δ 1.58 (3 H, d, J=5 Hz, CH₃-CH), 1.80 (4 H, m, CH₂-CH₂), 4.24 (4 H, m, 2 x CH₂-O), 5.7-6.7 (6 H, m, 2 x CH=CH₂), 6.87 (1 H, k, J=5 Hz, CH-CH₃).

Preparation 14

a) Methylene bis(3,3-dimethoxypropionate)

Cesium 3,3-dimethoxypropionate (19.95 g, 75 mmol) is added to dry DMF (1000 ml). Diiodomethane (10.04 g, 37.5 mmol) is added to the suspension and the reaction mixture is stirred for 2 days at 60°C under a dry N₂ atmosphere. DMF is removed under reduced pressure (0.01 mmHg). Diethyl ether (500 ml) is added to the residue, which is then washed with saturated aqueous sodium hydrogen carbonate (250 ml). The aqueous layer is extracted with diethyl ether (5 x 75 ml). The combined ether extracts are washed with water (2 x 100 ml), dried (MgSO₄) and evaporated to give 7.1 g (72%) product. ¹H NMR (300 MHz, CDCl₃): δ 2.61 (CH₂, d), 3.26 (CH₃, s).

b) Methylene bis(3-methoxypropenoate)

Methylene bis(3,3-dimethoxypropionate) (14.01g, 50 mmol) prepared as described in (a) above and a catalytic amount of p-toluene sulfonic acid is added to toluene (250 ml). The methanol is removed by warming the reaction under an N₂ atmosphere. When the reaction is complete the toluene is distilled off under reduced pressure. Diethyl ether (250 ml) is added and the mixture is washed with saturated aqueous sodium hydrogen carbonate (5x50 ml) and water (3x50 ml). The organic layer is dried (MgSO₄) before evaporation to give 8.52g

(79%) product. ^1H NMR (300 MHz, CDCl_3): δ 3.65 (2 x CH_3 , s), 5.2 (2 x CH, d), 5.8 ($\text{O}-\text{CH}_2-\text{O}$), 7.65 (2 x CH_2 , d).

Preparation 15

5 a) Methylene bis(10-undecenoate)

10-Undecylenic acid (12.75 g, 75 mmol) is dissolved in 100 ml water. Cesium carbonate (13.04 g, 40 mmol) is added to the mixture. The water is removed under reduced pressure and the salt dried for 2 hours in vacuo. The cesium salt is mixed with 150 ml DMF and diiodomethane is added to the solution. The reaction is stirred for 3 days at 60°C under an N_2 atmosphere. DMF is then removed under reduced pressure. The residue is purified through silica gel with hexane/ ethyl acetate (8:2) as eluant. The solvent is evaporated to give 7.18 g (54%) product. ^1H NMR (300 MHz, CDCl_3): δ 1.2-1.4 (10 x CH_2 , m), 1.6 (2 x CH_2 , m), 2.0 (2 x CH_2 , m), 2.19 (2 x CH_2 , t), 4.9 (2 x $\text{H}_2\text{C}=\text{}$, m), 5.88 ($\text{O}-\text{CH}_2-\text{O}$, s), 5.9 (2 x $\text{HC}=\text{}$, m). ^{13}C NMR (300 MHz, CDCl_3): δ 24.92-33.98 (8 x CH_2), 79.04 ($\text{O}-\text{CH}_2-\text{O}$), 114.18 ($=\text{CH}_2$), 139.11 ($=\text{CH}$), 172.48 ($\text{C}=\text{O}$).

b) Methylene bis(10-epoxyundecanoate)

Methylene bis(10-undecenoate) (8.8g, 25 mmol) prepared as described in (a) above is added under an N_2 atmosphere to methylene chloride and cooled to 0°C. Metachloroperbenzoic acid 55% (15.75g, 50 mmol) is added to methylene chloride (150 ml) and the organic layer is separated and dried (MgSO_4). The metachloroperbenzoic acid is then added dropwise to the diester. After completed addition the temperature is increased to 25°C. After 5 hours the reaction is complete. The mixture is washed with saturated aqueous sodium sulphite (75 ml) and saturated aqueous sodium hydrogen carbonate (2 x 75 ml). The organic layer is purified on neutral aluminium oxide. The solvent is removed under reduced pressure to yield 8.45g (82%) product. ^1H NMR (300 MHz, CDCl_3): δ

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1.2-1.7 (14 x CH₂, m), 2.35 (2 x CH₂CO, t), 2.45 (2 x CH, q),
2.75 (2 x CH, q), 2.90 (2 x CH, m), 5.75 (O-CH₂-O). ¹³C
NMR (300 MHz, CDCl₃): δ 24.58 (CH₂), 25.99 (CH₂), 28.94
(CH₂), 29.09 (CH₂), 29.32 (2 x CH₂), 32.45 (CH₂), 33.92
5 (CH₂), 47.06 (CH₂-O), 52.36 (CH-O), 79.06 (O-CH₂-O),
172.2 (C=O).

Preparation 16

Methylene bis(4-epoxypentanoate)

10 Metachloroperbenzoic acid (15.68 g, 55%, 50 mmol)
is dissolved in methylene chloride (200 ml). Water is
separated and the organic layer is dried (MgSO₄). The
resulting metachloroperbenzoic acid solution is added
dropwise to methylene bis(4-pentenoate) (4.10 g, 19
15 mmol) dissolved in methylene chloride (50 ml). The
mixture is stirred at ambient temperature under nitrogen
for 12 hrs, whereafter the reaction mixture is washed
with saturated aqueous sodium bicarbonate solution (50
ml), water (50 ml), dried (MgSO₄) and evaporated to give
20 3.61g (78%) of the title compound as a crystalline
product. ¹H NMR (300 MHz, CDCl₃): δ 1.70-1.85 (2xCH, m),
1.95-2.10 (2x CH, m), 2.50-2.55 (2xCH, 2xCH₂, m), 2.75
(2xCH, t), 3.0 (2xCH, m), 5.8 (O-CH₂-O, s). ¹³C NMR (75
MHz, CDCl₃): δ 27 (2xCH₂), 30 (2xCH₂), 47 (2xCH₂), 51
25 (2xCH), 79.8 (O-CH₂-O), 171.8 (2xC=O).

Preparation 17

Methylene bis(2-butenate)

30 Vinylacetic acid (4.3 g, 50 mmol) is added to an
aqueous cesium carbonate solution (50 ml). The mixture
is stirred for 5 min. and then evaporated, and the
residue is dried under vacuum for 2 hrs. The resulting
cesium salt and diiodomethane are added to
dimethylformamide (200 ml) and the mixture is stirred
35 for 24 hrs. at 50°C under nitrogen, whereafter the
dimethylformamide is removed under reduced pressure.
The residue is dissolved in diethyl ether (100 ml) and

washed with saturated aqueous sodium bicarbonate (25 ml) and water (25 ml). The organic layer is dried (MgSO_4) and evaporated to give 1.32 g (29%) product. ^1H NMR (300 MHz, CDCl_3): δ 1.9 (2x CH_2 ,m), 5.8-5.9 (2xCH,m), 5.9 (OCH₂O,s), 7.0-7.1 (2xCH,m).

Preparation 18

Methylene bis(chloroacetate)

Chloroacetic anhydride (12.75 g, 75 mmol), paraformaldehyde (2.25 g, 75 mmol) and conc. sulfuric acid (15 drops) are added to methylene chloride (15 ml). The mixture is stirred for 24 hrs. at 50°C under nitrogen, whereafter the reaction mixture is extracted with saturated aqueous potassium carbonate until carbon dioxide emission ends. The organic layer is dried (MgSO_4), evaporated to dryness and the residue is distilled (80°C, 0.15 mmHg) to yield 10.2 g (57%) product. ^1H NMR (200 MHz, CDCl_3): δ 4.1 (2xCH₂Cl,s), 5.9 (CH₂,s). ^{13}C NMR (200 MHz, CDCl_3): δ 41.1 (CH₂Cl), 81.4 (O-CH₂-O), 166.4 (CO).

Preparation 19

Methylene bis(4-oxopentanoate)

4-Oxopentanoic acid (11.6 g, 100 mmol) is dissolved in acetonitrile (70 ml), and 1,8-diazabicyclo[5.4.0]undec-7-ene (15.25 g, 100 mmol) diluted with acetonitrile (30 ml) is added. Diiodomethane (13.4 g, 50 mmol) is added in one batch, and the reaction mixture is refluxed under a nitrogen atmosphere. After 2 hours, gas chromatography indicates full consumption of diiodomethane. The solvent is removed in vacuo and the residual brown oil is transferred to a separation funnel with ethyl acetate (200 ml) and water (75 ml). The organic phase is washed with 1M sodium bicarbonate (25 ml) and water (3 x 25 ml), dried over MgSO_4 , and the solvent is removed in vacuo to yield the title compound (10 g). ^1H NMR: δ 2.19

(2 x CH₃, s), 2.760-2.804 (2 x CH₂, t), 2.600-2.645 (2 x CH₂, t), 5.735 (CH₂ bridge, s).

Preparation 20

Methylene bis(succinimidylazelaate)

5 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide
hydrochloride (1.49 g, 7.71 mmol) was added in portions
to a stirred solution of methylene bis(hydrogen azelaate)
from Example 25 (1.00 g, 2.57 mmol) and N-
10 hydroxysuccinimide (0.89 g, 7.71 mmol) in dry
dimethylformamide at ambient temperature. After 20
hours stirring, the reaction mixture was poured into
ice-water and the product precipitated as an oil. The
colourless oil was dissolved in diethylether (50 ml),
washed with water (3x10 ml) and dried over MgSO₄. The
15 solvent was removed under reduced pressure and hexane (5
ml) was added to the oily product. After seven days
storage at 4°C the oil had crystallized to a white, waxy
solid. Yield: 1.50 g (69%). m.p.: 45-47°C. ¹³C NMR (75
MHz, CDCl₃) δ: 24.42, 24.46, 25.59, 28.48, 28.63, 30.85,
20 33.82, 79.61, 168.6, 169.30, 172.34.

Preparation 21

Methylene bis(sulphosuccinimidylazelaate) sodium salt

25 Methylene bis(hydrogen azelaate) (0.38 g, 1 mmol),
N-hydroxysuccinimide sodium salt (0.48 g, 2.2 mmol) and
dicyclohexylcarbodiimide (0.45 g, 2.2 mmol) were
dissolved in dimethylformamide (10 ml). The suspension
was stirred overnight at room temperature under an
atmosphere of nitrogen. The reaction mixture was
30 filtered and purified by reversed phase chromatography
(RP-8) with water/acetonitrile (1:1) as eluant to give
the title compound.

Preparation 22

35 a) Methylene bis(10,11-dihydroxyundecanoate)

N-Methylmorpholine-N-oxide (13.5 g, 11 mmol) and
methylene bis(10-undecenoate) from Preparation 15(b) (19

g, 5 mmol) were dissolved in 400 ml of a mixture of tetrahydrofuran and water (3:1 v/v). A catalytic amount of osmium tetroxide was added, and the solution stirred at ambient temperature for 20 hours. TLC indicated complete consumption of the starting material. Excess sodium hydrogen sulphite and sodium chloride were then added to the reaction mixture. The product was extracted from the resulting mixture with ethyl acetate (400 ml) and the water phase was washed with ethyl acetate (3 x 50 ml). The combined organic phases were dried and evaporated, and the product recrystallised from tetrahydrofuran to yield 14.5g (68%) of the product as a white solid. ^{13}C NMR (45 MHz) CD_3OD : δ 24.6-34.0 (16 x CH_2), 66.6 (2 x CH_2OH), 72.3 (2 x CHOH), 79.2 ($\text{O}-\text{CH}_2-\text{O}$), 174.0 (2 x $\text{C}=\text{O}$).

b) Methylene bis(10-oxodecanoate)

Methylene bis(10,11-dihydroxyundecanoate) (2.24 g, 5 mmol) was dissolved in 150 ml tetrahydrofuran. Sodium metaperiodate (2.06 g, 10 mmol) was dissolved in 150 ml water and added dropwise to the tetrahydrofuran solution. TLC indicated full consumption of the diol after 60 minutes, whereupon sodium chloride was added to the reaction mixture until the two phases separated. The water phase was extracted with diethyl ether (3 x 50 ml). The combined organic phases was dried with magnesium sulphate and evaporated to give the title product as an oil, 1.43 g (74%). ^{13}C NMR (45 MHz) CDCl_3 : δ 21.9-43.9 (16 x CH_2), 79.1 ($\text{O}-\text{CH}_2-\text{O}$), 173.0 (2 x $\text{C}=\text{O}$), 202.6 (2 x CHO).

Example 1

1. Gas-filled albumin microspheres are prepared according to EP-A-0359 246 and resuspended to homogeneity by gentle rolling on a vial roller.

- 25 -

2. 25 ml of the suspension are poured into a 25 ml separating funnel and left for 30 min. The bottom 20 ml are discarded.
- 5 3. To the remaining 5 ml is added 20 ml of a phosphate buffer (20 mM NaPO_4 , pH 7.0), and the resulting suspension is transferred to a vial with a cap septum.
- 10 4. The vial is centrifuged upside down at 170 x g for 5 min.
- 15 5. The solution underneath the microsphere layer is withdrawn using a syringe, and the microspheres are resuspended in 25 ml of the phosphate buffer by 10 min of gentle rolling.
6. Points 4 and 5 are repeated twice.
- 20 7. The resulting suspension is centrifuged as in point 4, and the microspheres are resuspended in the phosphate buffer to a final concentration of about 5×10^8 particles per ml.
- 25 8. The crosslinker methylene bis(α -formylacetate), prepared as described in Preparation 1, is added to the suspension, and the crosslinking reaction is allowed to proceed for the desired time (usually 30-60 min) under gentle rolling.
- 30 9. 1.5 M Tris-HCl-buffer (pH 8.8) is added to a final concentration of 0.25 M, and the suspension is rolled gently for 10 min.
- 35 10. The vial is centrifuged as in point 4, and the solution underneath the microsphere layer is removed as in point 5.

11. The microspheres are resuspended in phosphate buffer (same volume as final volume in point 9), and the suspension is rolled for 10 min.
- 5 12. Points 10 and 11 are repeated twice.
13. The resulting suspension is centrifuged as in point 4, and the microspheres are resuspended in the phosphate buffer to a final concentration of about
10 5×10^8 particles per ml.
14. This final suspension of crosslinked gas/albumin microspheres is stored at 4°C.

15 Example 2-22

The procedure of Example 1 is repeated using crosslinking agents prepared as described in Preparations 2-22, except that dimethyl sulphoxide is
20 used in place of phosphate buffer in the processing of the gas-filled albumin microspheres according to steps 3-7 and the crosslinking agent is added in step 8 as a solution in dimethyl sulphoxide.

25 The number and size distribution of the products are determined by Coulter counter analysis.

CLAIMS

1. Contrast agents for use in diagnostic ultrasound studies comprising microbubbles of gas or a gas precursor encapsulated in a protein shell characterised in that the said protein is crosslinked with biodegradable crosslinking groupings.
2. Contrast agents as claimed in claim 1 wherein the crosslinking groupings contain biodegradable linkages selected from amide, imide, imine, ester, anhydride, acetal, carbamate, carbonate, carbonate ester and disulphide groups.
3. Contrast agents as claimed in claim 2 wherein the crosslinking groups contain biodegradable linkages of formula
- $$-(Y)_n-CO-O-C(R^1R^2)-O-CO-(Z)_n-$$
- (where Y and Z, which may be the same or different, are -O-, -S- or -NR³-; R¹ and R², which may be the same or different, are hydrogen atoms or carbon-attached monovalent organic groups or together represent a carbon-attached divalent organic group; R³ is a hydrogen atom or an organic group; and the symbols n, which may be the same or different, are zero or 1).
4. Contrast agents as claimed in any of the preceding claims wherein the protein is albumin, gelatin or γ -globulin.
5. Contrast agents as claimed in claim 4 wherein the protein is human serum albumin.
6. Contrast agents as claimed in any of the preceding

claims further containing an inorganic particulate stabiliser.

7. A process for the preparation of a contrast agent
5 as claimed in claim 1 which comprises encapsulating a gas or gas precursor in a protein and crosslinking the protein with biodegradable crosslinking groups before, during or after said encapsulation.

10 8. A process as claimed in claim 7 wherein crosslinking is effected after encapsulation.

9. A process as claimed in claim 7 or claim 8 wherein crosslinking is effected using a crosslinking agent of
15 formula (I)



(where X is a linking group containing one or more biodegradable linkages as defined in claim 2 or claim 3
20 and A¹ and A², which may be the same or different, are functional groups reactive with proteins).

10. A process as claimed in claim 9 in which A¹ and A² are both aldehyde groups.
25

11. A process as claimed in any of claims 8 to 10 wherein encapsulation is effected by agitation or sonication of the protein in an aqueous medium to yield a protein foam which is dried and thereafter suspended
30 in a solution of the crosslinking agent in a polar organic solvent.

12. A process as claimed in claim 11 in which the crosslinking agent is a compound of formula (I) as
35 defined in claim 9 in which A¹ and A² are both O-linked sulphonated N-hydroxysuccinimidyl residues.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 92/00716

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC Int.C1.5 A 61 K 49/00																	
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%; border-bottom: 1px solid black; padding: 5px;">Classification System</td> <td style="border-bottom: 1px solid black; padding: 5px;">Classification Symbols</td> </tr> <tr> <td style="padding: 5px;">Int.C1.5</td> <td style="padding: 5px;">A 61 K</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched⁸</div>			Classification System	Classification Symbols	Int.C1.5	A 61 K											
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III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; padding: 5px;">Category *</th> <th style="width: 70%; padding: 5px;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%; padding: 5px;">Relevant to Claim No.¹³</th> </tr> </thead> <tbody> <tr> <td style="vertical-align: top; padding: 5px;">P, Y</td> <td style="padding: 5px;">EP, A, 0441468 (SCHERING AG) 14 August 1991, see claims 1-5, 8, 9 ---</td> <td style="vertical-align: top; padding: 5px;">1-12</td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">The Journal of the Acoustical Society of America, vol. 87, no. 4, April 1990, Acoustical Society of America, (New York, US), H.J. BLEEKER et al.: "Ultrasonic characterization of Albunex, a new contrast agent", pages 1792-1797, see page 1796: "Conclusion" ---</td> <td style="vertical-align: top; padding: 5px;">1-12</td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">X, Y</td> <td style="padding: 5px;">EP, A, 0224934 (S.B. FEINSTEIN) 10 June 1987, see claims 1-10 (cited in the application) ---</td> <td style="vertical-align: top; padding: 5px;">1-12</td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">P, X</td> <td style="padding: 5px;">WO, A, 9204392 (HOLMES) 19 March 1992, see claims 1, 10, 11; page 4, lines 14-36; page 8, line 18 - page 9, line 3 --- -/-</td> <td style="vertical-align: top; padding: 5px;">1-12</td> </tr> </tbody> </table>			Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	P, Y	EP, A, 0441468 (SCHERING AG) 14 August 1991, see claims 1-5, 8, 9 ---	1-12	Y	The Journal of the Acoustical Society of America, vol. 87, no. 4, April 1990, Acoustical Society of America, (New York, US), H.J. BLEEKER et al.: "Ultrasonic characterization of Albunex, a new contrast agent", pages 1792-1797, see page 1796: "Conclusion" ---	1-12	X, Y	EP, A, 0224934 (S.B. FEINSTEIN) 10 June 1987, see claims 1-10 (cited in the application) ---	1-12	P, X	WO, A, 9204392 (HOLMES) 19 March 1992, see claims 1, 10, 11; page 4, lines 14-36; page 8, line 18 - page 9, line 3 --- -/-	1-12
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X, Y	EP, A, 0224934 (S.B. FEINSTEIN) 10 June 1987, see claims 1-10 (cited in the application) ---	1-12															
P, X	WO, A, 9204392 (HOLMES) 19 March 1992, see claims 1, 10, 11; page 4, lines 14-36; page 8, line 18 - page 9, line 3 --- -/-	1-12															
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>																	
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="text-align: center; padding: 5px;">26-05-1992</td> <td style="text-align: center; padding: 5px;">29. 07. 92</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">International Searching Authority</td> <td style="border-bottom: 1px solid black; padding: 5px;">Signature of Authorized Officer</td> </tr> <tr> <td style="text-align: center; padding: 5px;">EUROPEAN PATENT OFFICE</td> <td style="text-align: center; padding: 5px;"> </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	26-05-1992	29. 07. 92	International Searching Authority	Signature of Authorized Officer	EUROPEAN PATENT OFFICE								
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International Searching Authority	Signature of Authorized Officer																
EUROPEAN PATENT OFFICE																	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		Relevant to Claim No.
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	
Y	Methods in Enzymology, vol. 172, part S, 1989, Academic Press, Inc., H.G. BAUMERT et al.: "[32] Cross-linking techniques", pages 584-609, see table I; pages 587, 588, 589 (cited in the application) -----	1-12

INTERNATIONAL SEARCH REPORT

INTERNATIONAL APPLICATION NO.

PCT/EP 92/00716

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: SEE REMARK!
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

In view to the large number of compounds which are comprised by the term "protein" shell of claim 1 the search has been limited to the compounds mentioned in the claims and examples for economic reasons. (GUIDELINES ART. 6...CHAPT. II.7, last sentence and CHAPT. III, 3.7).

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

EP 9200716
SA 57670

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 16/07/92.
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0441468	14-08-91	DE-A- 4004430	14-08-91
		AU-A- 7098291	17-10-91
EP-A- 0224934	10-06-87	US-A- 4718433	12-01-88
		AU-B- 575735	04-08-88
		AU-A- 6609786	11-06-87
		DE-A- 3683735	12-03-92
		JP-B- 3041168	21-06-91
		JP-A- 62181033	08-08-87
		US-A- 4774958	04-10-88
WO-A- 9204392	19-03-92	AU-A- 8525691	30-03-92

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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